DESCRIPTION

Use of Asparaginase and Glutaminase to Treat Autoimmune Disease and Graft Versus Host Disease

Related Applications

This application claims priority to U.S. Patent Application Serial No. 09/094,435, by Donald L. Durden, entitled "Utilization of *Wolinella succinogenes* asparaginase in the treatment of human hematologic and autoimmune disease" (Lyon & Lyon Docket No. 234/274), filed June 9, 1998, which claims priority to U.S. provisional patent application 60/049,085, filed June 9, 1997.

Field Of Invention

The present invention relates to methods for the utilization of recombinant microbial enzymes, including asparaginases and glutaminases, in the treatment of autoimmune diseases and Graft versus Host disease.

Background Of Invention

The references cited below are not admitted to be prior art to the inventions described herein.

Juvenile rheumatoid arthritis (JRA) is the most common rheumatic condition of childhood. Recent long-term follow-up studies have shown that JRA is not benign and the proportion of patients with a favorable outcome is less than initially thought (Wallace, 1991; Levinson, 1992). Approximately one-third of all patients achieve adequate control of their disease with nonsteroidal anti-inflammatory drugs (NSAIDs), but the remainder of patients are candidates for more aggressive therapy with second-line agents.

Placebo-controlled trials and long-term prospective studies in children with JRA showed a lack of efficacy among agents such as penicillamine, hydroxychloroquine, oral gold, and intravenous immune globulin. Brewer, 1986; Giannini,

1993; Silverman, 1993. Secondary treatment failures even with new standard medications such as methotrexate are common, creating a high demand for new safe and effective agents in these refractory diseases.

Asparaginases are used as front-line therapy in the treatment of acute leukemia. Enzymes that deplete asparagine or glutamine possess immunosuppressive effects and have been shown to have anti-inflammatory properties. However, the mode of action and the final lethal route of susceptible cells deprived of L-asparagine or L-glutamine is still undetermined.

The clinically utilized forms of L-Asparaginase are immunogenic proteins derived either from *E. coli* (EC), *Erwinia carotovora,* or *Wolinella succinogenes* (WS). *E coli* possesses two asparaginase enzymes, one constitutive and another induced by anaerobic conditions. The asparaginase induced by anaerobic conditions is known to have a tumor inhibitory effect. Interestingly, L-Asparaginase from *E. coli* has cytotoxic, but also immunosuppressive, properties due to its glutamine depleting effect. In fact, the immunosuppressive effect of L-Asparaginase has been attributed to this glutaminase property of this enzyme. The EC asparaginase has recently been covalently modified using polyethylene glycol (PEG) conjugation, to form PEG asparaginase, to reduce antigenicity and extend the half-life of the EC enzyme.

Unlike other anti-tumor agents (cyclophosphamide, etoposide, *etc.*), asparaginases from *E. coli* (EC and EC-PEG) are not mutagenic, and not associated with second malignancy. At the same time, EC and EC-PEG enzymes are not myelosuppressive. Hence, patients treated with asparaginase are not at risk for development of sepsis or other severe life threatening conditions, for example, infections.

EC and EC-PEG have potent antileukemic activity and cause minimal toxicity in children. The limited toxicity of these enzymes is restricted to rare coagulation abnormalities in less than 1% of patients, which can be

managed easily. Mild allergic reactions have also been described.

The immunosuppressive effects of EC are restricted to its effects on the lymphoid system. L-Asparaginase derived from *E. coli* suppresses the humoral or cell-mediated immunological response to T cell-dependent immunogens on sheep red blood cells. The EC enzyme inhibits T-cell immunity to the antigen, SRBC, as measured by antibody titer, ADCC, and immunoglobulin producing cells in the spleen (80% reduction). The effects of *E. coli* asparaginase treatment on spleen histology and lymphocyte populations are known to include a marked reduction in the size and reactivity of the germinal centers, which correlates with a marked reduction in the cytoplasmic immunoglobulin-containing cells (B-cell immunoblasts).

These data support the hypothesis that depletion of glutamine, or asparagine together with glutamine, after treatment with *E. coli* asparaginase results in marked immune suppression. In contrast, asparagine deprivation alone, caused by the administration of the glutaminase-free asparaginase from *WS*, does not affect spleen histology or lymphocyte marker distribution and is not immunosuppressive.

Definition Of Terms

Unless otherwise expressly defined, the terms used herein will be understood according to their ordinary meaning in the art, although the following terms will be understood to have the following meanings, unless otherwise indicated.

An "analog" of a protein, *e.g.*, asparaginase or glutaminase, refers to a polypeptide that differs in some way from its form(s) found naturally. For example, in certain embodiments, an analog of asparaginase or glutaminase will refer to an enzyme wherein one or more amino acids has been deleted from the naturally occurring amino acid sequence. Alternatively, one or more amino acid

residues may be substituted with a different amino acid. Other analogs include those wherein additional amino acids have been added to the native sequence. For example, one or more amino acids may be added to the amino terminus and/or carboxy-terminus of the enzyme, or be inserted between internal amino acid residues. Such analogs can be prepared by any suitable technique, although modifying a recombinant gene to encode the desired change(s) will typically be employed. Other analogs include those wherein one or more amino acid residues are derivatized, *e.g.*, glycosylated, pegylated, acylated, or otherwise bound covalently to a molecule not attached to native form(s) of the protein. Of course, analogs according to the invention include those where an amino acid residue is added to or substituted in the native amino acid sequence, and this new residue is itself later modified, for example, by a covalent modification performed after the enzyme has been at least partially purified or isolated. Moreover, as used herein, an asparaginase or glutaminase analog includes those that have been modified and exhibit altered biochemical or physiological properties, *e.g.*, different substrate specificity and/or affinity, altered quarternary structure, *etc.* After generating analogs, *e.g.*, by a rational design strategy, random mutagenesis, *etc.*, the proteins can be screened for biological activity, as described elsewhere herein. When large numbers of analogs are generated, high throughput screening methods are preferred in order to identify analogs having the desired characteristics. Those analogs found to exhibit the desired activity *in vitro* may then be tested *in vivo* for activity and pharmacokinetic properties.

A "unique contiguous amino acid sequence" means an amino acid sequence not found in a naturally occurring protein or polypeptide. Thus, a "unique contiguous amino acid sequence of *Wolinella succinogenes*", for example, refers to a sequence which contains one or more amino acid

substitutions, insertions, or deletions, as compared to corresponding region of the native enzyme.

A "disease which responds to asparagine or glutamine depletion" refers to a disorder wherein the cells responsible for or otherwise correlates with the disease state either lack or have a reduced ability to synthesize, uptake, or otherwise utilize asparagine or glutamine. Depletion or deprivation of asparagine to such cells can be partial or substantially complete, so long as the desired therapeutic benefit is achieved. In certain embodiments, more than about 50% of asparagine or glutamine in the serum is depleted, preferably greater than about 75%, with depletion of more than 95% being most preferably achieved. Representative examples of diseases that respond to asparagine or glutamine depletion or deprivation include certain non-hematologic diseases. Non-hematologic diseases associated with asparagine or glutamine dependence include autoimmune diseases, for example rheumatoid arthritis, systemic Lupus erythematosus (SLE), autoimmunity, collagen vascular diseases, AIDS, *etc.* Other autoimmune diseases that may be treated according to the instant methods include, without limitation, osteo-arthritis, Issac's syndrome, psoriasis, insulin dependent diabetes mellitus, multiple sclerosis, sclerosing panencephalitis, systemic lupus erythematosus, rheumatic fever, inflammatory bowel disease (*e.g.*, ulcerative colitis and Crohn's disease), primary billiary cirrhosis, chronic active hepatitis, glomerulonephritis, myasthenia gravis, pemphigus vulgaris, and Graves' disease. Notwithstanding the foregoing, any disease the cells responsible for which respond, *e.g.*, cease proliferating, become senescent, undergo apotosis, die, *etc.*, to asparagine or glutamine depletion may be treated in accordance with the instant methods. As those in the art will appreciate, cells suspected of causing disease can be tested for asparagine or glutamine dependence in any suitable *in vitro* or *in vivo* assay, *e.g.*, an *in vitro* assay wherein the growth medium lacks asparagine or glutamine.

A "patient" refers to an animal afflicted with a disease that responds to asparagine or glutamine depletion. Typically, patients treated in accordance with the instant methods are mammals, *e.g.*, bovine, canine, equine, feline, ovine, porcine, and primate animals, particularly humans.

An "expression vector" refers to a nucleic acid, typically a plasmid, into which heterologous genes of interest may be cloned and subsequently expressed. For expression, such vectors are generally introduced into a suitable host cell or population of host cells. The expression vector can be introduced by any appropriate technique. Preferred techniques include transformation, electroporation, transfection, and ballistic (*e.g.*, "gene gun") introduction. Depending upon the vector employed, suitable host cells for expression of the desired heterologous gene(s) include prokaryotic and eukaryotic cells. Preferred prokaryotic cells are transformation-competent bacterial cells such as *E. coli* strain and DH5α and JM 109. Preferred eukaryotic host cells include yeast and mammalian cell lines. As those in the art will appreciate, the particular expression vector/host cell system selected for expression of the desired heterologous gene depends on many factors, and is left to the skilled artisan to determine in the particular circumstances. Similarly, the conditions required for expression of the desired gene from an expression vector carrying the same depends on many factors, including the host cell type, the promoter(s) and other transcription regulation elements employed, the media (or medium) used, *etc.* Again, the selection made in a given circumstance is at the discretion of the artisan involved, and the particular employed is readily within the skill of such a person given the disclosure herein.

A protein that is "biologically active" is one that has at least one of the biological activities of the corresponding native protein, although the activity exhibited may differ in degree from that of the native

protein. For example, an analog of *W. succinogenes* asparaginase according to the invention may have a greater specific activity, longer serum half-life, *etc.* than the native form of the protein.

A protein that has an "epitope-tag" refers to a protein having one or more, preferably two or more, additional amino acids covalently attached thereto or incorporated therein. The tag has a distinct epitope that can be recognized by another protein, *e.g.*, an antibody that binds that epitope, preferably with high affinity; or a protease that cleaves in or around a specific amino acid sequence (*e.g.*, DAPI, cathepsin-C), *etc.* For example, as used herein an "N-terminal epitope tag" can refer to a peptide attached to the N-terminus of a protein, where the peptide has a conformation recognized by a particular antibody. Such a peptide and its corresponding antibody(ies) can be used to rapidly purify the polypeptide to which the peptide is attached by standard affinity chromatography techniques. Such antibodies, and any others used in the practice of this invention (*e.g.*, for targeting gene delivery vehicles), can be prepared used techniques widely known in the art. For example, *see* Harlow and Lane in *Antibodies, a Laboratory Manual*, Cold Spring Harbor Laboratory, 1988. Epitope tags may also be included at the C-terminus of the protein, and in internal regions where insertion of such a tag does not substantially and adversely affect the biological activity or pharmacokinetic properties of the enzyme.

A "therapeutically effective amount" of a protein (*e.g.*, an asparaginase, a glutaminase, or an analog thereof) means that amount required to produce the desired therapeutic effect. Of course, the actual amount required depends on many factors, such as the disease to be treated, the progression of the disease, and the age, size, and physical condition of the patient, as discussed in more detail below.

By "altering a pharmacokinetic property of a protein" is meant that a property of a drug as it acts in the body

over a period of time, *e.g.*, serum half-life, clearance rate, biodistribution, immunogenicity, *etc.*, is changed. Such alteration can be either an increase or decrease in the property being examined.

Summary Of Invention

One aspect of the present invention is directed to methods for the therapeutic utilization of native and/or recombinant forms of asparaginases and glutaminases in the treatment of diseases which respond to asparagine and/or glutamine depletion, including various autoimmune diseases which respond to asparagine and/or glutamine depletion. In preferred embodiments, these methods involve administering to a patient a therapeutically effective amount of a *W. succinogenes* asparaginase or glutaminase, an analog of either, or an acylated asparaginase or glutaminase derived from an organism other than *W. succinogenes*. Other asparaginases or glutaminases specifically envisioned include those from other fungal and bacterial sources, and include, but are not limited to, both recombinant and native asparaginases from *Wolinella succinogenes,* and recombinant and native asparaginases/glutaminases from *E. coli*, *Acinetobacter,* and *Erwinia*, for example.

Representative diseases that can be treated in accordance with the instant invention include autoimmune diseases, for example, arthritis (*e.g.*, rheumatoid arthritis), systemic lupus erythematosus (SLE), diabetes, and AIDS. The methods of the invention may also be used to treat Graft versus Host Disease, for example. Typically, the instant methods will be applied to humans afflicted with a disease which responds to asparagine and/or glutamine depletion, although other patient classes, particularly mammals (*e.g.*, bovine, canine, equine, feline, ovine, porcine, and primate animals) suffering from a disease which responds to asparagine and/or glutamine depletion can be similarly treated.

Methods for isolating native *W. succinogenes* asparaginase, producing recombinant *W. succinogenes* asparaginase *in vitro* or *in vivo*, making derivatives, analogs, and covalent modifications thereof, and making pharmaceutical formulations therefrom were described previously in U.S. Patent application Serial No. 09/094,435, by Donald L. Durden, entitled "Utilization of *Wolinella succinogenes* asparaginase in the treatment of human hematologic and autoimmune disease" (Lyon & Lyon Docket No. 234/274), filed June 9, 1998, incorporated by reference herein in its entirety including any drawings, tables, or figures. These methods can be applied analogously to asparaginases and glutaminases from other organisms, including those from other bacterial and fungal sources, including, but not limited to, recombinant and native asparaginases/glutaminases from *E. coli*, *Acinetobacter*, and *Erwinia*.

The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein. For example, in the methods of the invention, patients can be mammals, but in some embodiments this may not include mice or rats. Similarly, although all asparaginases and glutaminases are envisioned in the methods of the invention, in some embodiments this may not include native *E. coli* asparaginase.

Other features and advantages of the invention will be apparent from the following figures, detailed description, examples, and claims.

Brief Description Of The Drawings

The present invention may be better-understood and its advantages appreciated by those individuals skilled in the relevant art by referring to the accompanying drawings wherein:

Figure 1: Illustrates the nucleotide sequences of the forward [SEQ ID NO. 1] and reverse [SEQ ID NO. 2] PCR primers used in the amplification of the genomic L-asparaginase sequences of *W. succinogenes*.

Figure 2: Agarose gel electrophoresis of propidium iodine-stained *W. succinogenes* genomic DNA (lanes 1 and 2) and a 1.0 kb DNA fragment derived from PCR amplification. Lanes 3 and 4 are DNA molecular weight markers. Lane 5 is the 1.0 kb *W. succinogenes*-specific PCR fragment amplified using the two PCR primers shown in Figure 1. Lane 6 contains a ϕX174 DNA molecular weight marker.

Figure 3: Restriction enzyme analysis of 4 colonies which were isolated following the ligation of the 1.0 kb *W. succinogenes*-specific PCR fragment into the PCR II vector. The 1.0 kb DNA was digested with BamH1 (lanes 2-5); EcoR1 (lanes 6-9); and BamH1 and EcoR1 (lanes 10-13). Lane 14 represents a DNA molecular weight ladder. The 1.0 kb *W. succinogenes*-specific DNA fragment is denoted by an arrow.

Figure 4: Agarose gel electrophoresis of the DNA fragments amplified from the selected, "positive" clones utilizing *W. succinogenes* asparaginase-specific primers. Lanes 1 and 7 are molecular weight markers. Lanes 2 and 4

represent DNA extracted from bacterial colonies #1 and #3 from lanes 2 and 4 of Figure 3. Lane 6 represents a sample of the *W. succinogenes* asparaginase PCR amplification product (amplified from *W. succinogenes* genomic DNA from Figure 2, lane 5) used in the initial ligation reaction. It should be noted that the fragment cloned into the PCR II vector was shown to be exactly the same size (*i.e.*, 1.0 b) as the initial PCR amplification product.

Figure 5: Illustrates the results of a determination of the anti-tumor activity of *W. succinogenes (WS)*, *E. coli* (EC) and *E. carotovora* (Erw) asparaginases against tumors generated by the subcutaneous injection of 6C3HED Gardner lymphosarcoma cells in C3H mice. Anti-tumor activity was measured as a function of caliper-measured tumor volume (cm^3). The negative control consisted of injections of 0.01 M phosphate buffer (pH 7.0) into C3H mice using the same injection schedule as for the asparaginases.

Figure 6: Illustrates the DNA sequence [SEQ ID NO. 3] of the modified *W. succinogenes* asparaginase-specific DNA insert. This sequence contains not only the coding sequence of the native *W. succinogenes* asparaginase (beginning with codon 40 of Figure 6 and not including the final 23 3' - terminal nucleotides of Figure 6), but also 39 codons for the N-terminal epitope "tag" shown in Figure 6.

Figure 7: Is a schematic representation of a chemical modification for a protein, for example *W. succinogenes* asparaginase.

Figure 8: Illustrates the lack of cross-reactivity between different dilutions of a patient's plasma known to contain high-titer neutralizing antibodies against *E. coli* asparaginase and the *W. succinogenes* enzyme.

Figure 9: Illustrates the lack of cross-reactivity between different dilutions of polyclonal high-titer neutralizing antibodies against *E. coli* asparaginase and asparaginase derived from *W. succinogenes*.

Figure 10: Demonstrates that *E. coli* asparaginase reverses established arthritis in CIA model. Digital image of mouse extremity before and after treatment with *E. coli* asparaginase. Mice were injected with bovine collagen type II in complete Freund's adjuvant on day 0 and boosted with same antigen on day +21. Arthritis developed on day +35 following immunization (Panel A) graded as 3+ arthritic involvement. Mouse treated with 50 IU of *E. coli* asparaginase daily for 1 week showed dramatic reversal of arthritic involvement from score of +3 to 0 on day + 42 as depicted in Panel B.

Figure 11: Demonstrates the effects of *E. coli* asparaginase on established arthritis in CIA mouse model. CIA was induced in DBA/1 mice as described above. On day +35 mice that developed detectable arthritis were separated into equivalent groups. One group received

E. coli asparaginase 50 IU/injection daily for 8 weeks the other group received PBS injections. Arthritic scores were compiled in blinded manner over the next 8 weeks of evaluation as depicted in bar graphs for two experimental groups. The data were analyzed for statistic significance. The difference between *E. coli* asparaginase and control PBS treated groups on months 1 and 2 was significant ($p < 0.05$).

Figure 12: Demonstrates the effects of *E. coli* asparaginase on established arthritis induced LPS/CIA model. CIA was induced in DBA/1 mice as described above. On day +21 mice were boosted with 100 ug collagen in Freuds adjuvant. On day +49 and +54 we administered LPS (40 µg/mouse IP). Mice developed LPS/CIA on day +61 and were separated into equal groups based on the arthritic scores. One group was treated with *E. coli* asparaginase 50 IU daily injections IP on Monday, Wednesday and Friday and other group was treated with PBS. Treatment was extended to 4 weeks. The bars represent the mean arthritic score over time. The data were evaluated by Student t-test and the differences observed between the *E. coli* asparaginase-treated mice on weeks 1-4 were statistically significant as compared to controls at ($p< 0.01$).

Detailed Description Of The Invention

Asparaginases and glutaminases can be used in the treatment of autoimmune diseases and Graft versus Host

Disease, and alter the natural course of autoimmunity. There is a dramatic clinical response to L-asparaginase in cancer treatment, although host toxicity and immuno-suppression also arise. The advantages to using L-asparaginase treatment for auto-immune and Graft versus Host diseases include the fact that immuno-suppression is a desired effect, and that lower and less frequent doses are likely to be required, limiting toxicity to the host.

Described herein are exemplary methodologies for the isolation of "native" asparaginases and glutaminases, as well as for the production (using recombinant expression vectors) of recombinant asparaginases and glutaminases and analogs thereof, *e.g.*, those which have been acylated and those which have been modified to include additional or alternate amino acids that have been acylated or otherwise modified (*e.g.*, by pegylation).

The following sections elaborate upon some of the various biochemical and physiological effects of clinical utilization of asparaginase or glutaminase therapy in the treatment of diseases associated with asparagine or glutamine dependence.

I. Review of the Clinical use of Asparaginase and Glutaminase

Asparaginases are enzymes which catalyze the deamidation of L-asparagine (asparaginase activity) and L-glutamine (glutaminase activity). *See* Cantor, P. S. & Schimmell, M. R., *Enzyme Catalysis, 2nd ed.,* (T. Pettersonn & Y. Tacashi, eds.) Sanders Scientific Press, New York pp. 219-23. (1990). L-glutamine serves as the amide donor in purine biosynthesis, as well as other transamination reactions, and hence plays a role in DNA and cyclic nucleotide metabolism.

In vivo biochemical activity of asparaginase was first documented to be present in guinea pig serum in 1922 (*see* Clementi, A., La desamidation enzmatique de l'asparagine chez les differentes especes-animals et la signification

physiologique de sa presence dass l'organisme, 19 *Arch. Intern. Physiol.* 369 (1922)). The subsequent discovery that asparaginase isolated from guinea pig serum was the active agent which inhibited the *in vivo* growth of certain asparagine-dependent mammalian tumors without concomitant deleterious effects on normal tissue (*see* Broome, J. D., Evidence that the asparaginase activity of guinea pig serum is responsible for its anti-lymphoma effects, 191 *Nature* 1114 (1961)) suggested that this enzyme could be utilized as an anti-neoplastic agent.

Because L-asparagine is a non-essential amino acid, asparaginase was initially thought to represent a unique prototype of selective chemotherapy in which treatment could be directed specifically and selectively against asparagine-dependent cells. However, the low levels of asparaginase in guinea pig serum necessitated the development of a more practical source of this enzyme.

Subsequently, microbial asparaginase isolated from *Escherichia coli* and *Erwinia carotovora* were shown to act as potent anti-leukemic agents (*see* Howard, J. B. & Carpenter, F.H., L-asparaginase from *Erwinia carotovora:* substrate specificity and enzymatic properties, 247 *J. Biol. Chem.* 1020 (1972); Campbell, H. A., et al., Two asparaginases from *Escherichia coli* B: their separation, purification, and anti-tumor activity, 6 *Biochemistry* 721 (1967)), and when one of these enzymes was utilized in combination with the chemotherapeutic agent vincristine and the corticosteroid prednisone for the treatment of acute lymphoblastic or acute undifferentiated human leukemia, an overall remission rate of 93% was reported (*see* Ortega, J.A., *et al.*, L-asparaginase, vincristine, and prednisone for the induction of first remission in acute lymphocytic leukemia, 37 *Cancer Res.* 535 (1977)).

While these asparaginases possess potent anti-leukemic activity, clinical utilization of the aforementioned microbial asparaginases resulted in a wide range of host toxicity (*e.g.*, hepatic, renal, splenic, pancreatic

dysfunction and blood coagulation) and pronounced immunosuppression (*see* Ohno, R. & Hersh, E. M., Immunosuppressive effects of L-asparaginase, 30 *Cancer Res.* 1605 (1970)), unlike asparaginase isolated from guinea pig serum (*see* Cooney, D.A., et al., L-asparaginase and L-asparagine metabolism, 10 *Ann. Rev. Pharmacol.* 421 (1970)).

Examination of the effects of *E. coli* asparaginase treatment on spleen histology and lymphocyte populations revealed a marked reduction in both the size and reactivity of the splenic germinal centers which was concomitantly associated with a marked reduction in the cytoplasmic immunoglobulin-containing cells (B-cell immunoblasts; *see* Distasio, J.A., *et al.*, Alteration in spleen lymphoid populations associated with specific amino acid depletion during L-asparaginase treatment, 42 *Cancer Res.* 252 (1982)). Additionally, examination of the lymphocyte sub-population within the spleen revealed that there was a 40% reduction in the percentage of surface immunoglobulin-expressing cells (B-cells) accompanied by an increase in the ratio of Thy-1.2-expressing cells (T-cells), whereas the ratio of Lyt-2 to Lyt-1 cells remained unchanged in comparison to the control animal group. These results supported the hypothesis that glutamine, or glutamine combined with asparagine depletion initially resulting from administration of *E. coli* asparaginase, caused a marked decrease in spleen lymphocytic cells of the B-cell lineage.

Another important adverse clinical effect associated with traditional microbial asparaginase treatment is hepatic dysfunction (*see* Schein, P.S., *et al.*, The toxicity of *E. coli* asparaginase, 29 *Cancer Res.* 426 (1969)). Patients treated with *E. coli* asparaginase generally exhibit decreased plasma levels of albumin, antithrombin III, cholesterol, phospholipids, and triglycerides. Other indications of asparaginase-induced hepatic dysfunction and pathology include fatty degenerative changes, delayed bromosulfophthalein clearance, and increased levels of serum glutamic-oxaloacetic transaminase and alkaline phosphatase.

Although some investigators have reported that low dosages of *E. coli* asparaginase result in limited hepatotoxic complications, sensitive indicators of hepatic function in some patients receiving low dosages, however, still reveals significant hepatic disease which may result in life-threatening coagulopathy (*see* Crowther, D., Asparaginase and human malignant disease, 229 *Nature* 168 (1971)).

The hepatotoxic effects of microbial asparaginases may be a result of their capability to hydrolyze both asparagine and glutamine. One biochemical difference between *E. coli* and *E. carotovora* asparaginases and the enzyme derived from guinea pig is the non-specific amidohydrolase activity associated with the microbial enzymes (*see* Howard, J.B. & Carpenter, F.H., (1972) *supra;* Campbell, H.A., et al., (1967) *supra*). For example, *E. coli* asparaginase has been shown to possess a 130-fold greater level of glutaminase activity as compared to the activity of *Wolinella succinogenes* (previously classified as *Vibrio succinogenes)* asparaginase. As a result, patients treated with the conventional microbial asparaginases show a marked reduction in serum levels of both glutamine and asparagine (*see* Schrek, R., *et al.*, Effect of L-glutaminase on transformation and DNA synthesis of normal lymphocytes, 48 *Acta Haematol.* 12 (1972)), which may demonstrate a possible correlation between glutamine deprivation and asparaginase-induced clinical toxicity (*see* Spiers, A.D.S., *et al.*, L-glutaminase/L-asparaginase: human pharmacology, toxicology, and activity in acute leukemia, 63 *Cancer Treat. Rep.* 1019 (1979)).

The relative importance of L-glutamine in mammalian intermediary metabolism served to stimulate further research into the possible role of glutamine deprivation in asparaginase-induced immunosuppression. Lymphoid tissue has been shown to have relatively low levels of glutamine synthetase activity (*see* El-Asmar, F.A. & Greenberg, D.H., Studies on the mechanism of inhibition of tumor growth by glutaminase, 26 *Cancer Res.* 116 (1966); Hersh, E.M., L-

glutaminase: suppression of lymphocyte blastogenic responses *in vitro,* 172 *Science* 139 (1971)), suggesting that these tissues may be particularly sensitive to the depletion of exogenous glutamine. In contrast, some investigators have proposed that asparagine depletion alone may be responsible for asparagine-induced immunosuppression (*see* Baechtel, F. S., *et al.*, The influence of glutamine, its decomposition products, and glutaminase on the transformation of human lymphocytes, 421 *Biochem. Biophys. Acta* 33 (1976)).

While the immunosuppressive effect of *E. coli* and *E. carotovora* asparaginases are well-documented (*see* Crowther, D., (1971) *supra;* Schwartz, R.S., Immunosuppression by L-asparaginase, 224 *Nature* 276 (1969)), the molecular biological basis of these functions have not yet been fully elucidated. The inhibition of lymphocyte blastogenesis by various L-glutamine antagonists (*see* Hersh, E.M. & Brown, B.W., Inhibition of immune response by glutamine antagonism: effect of azotomycin on lymphocyte blastogenesis, 31 *Cancer Res.* 834 (1980)) and glutaminase from *Escherichia coli* (*see* Hersh, E.M., (1971) *supra*) tends to be illustrative of a possible role for glutamine depletion in immunosuppression. It has also been demonstrated that inhibition of the lymphoid blastogenic response to phytohemagglutinin (PHA) by *E. coli* asparaginase can be reversed by the addition of L-glutamine, but not by the addition of L-asparagine. *See* Simberkoff, M.S. & Thomas, L., Reversal by L-glutamine of the inhibition of lymphocyte mitosis caused by *E. coli* asparaginase, 133 *Proc. Soc. Exp. Biol. (N. Y.)* 642 (1970). Additionally, a correlation between immunosuppression and the relative amount of glutaminase activity has been suggested by the observation that *E. carotova* asparaginase is more effective than *E. coli* asparaginase in suppressing the response of rabbit leukocytes to PHA (*see* Ashworth, L.A.E. & MacLennan, A.P., Comparison of L-asparaginases from *Escherichia coli* and *Erwinia carotovora* as immunosuppressant, 34 *Cancer Res.* 1353 (1974)). However, the significance of these *in vitro* studies is limited

because the *in vivo* fates of asparaginases and the homeostatic control of asparagine and glutamine may result in a modification of the immunosuppressive effects of anti-neoplastic asparaginases.

Another significant problem associated with the use of microbial asparaginases is that patients treated with *E. coli* and *E. carotovora* asparaginases frequently develop neutralizing antibodies of the IgG and IgM immunoglobulin class (*see, e.g.*, Cheung, N. & Chau, K., Antibody response to *Escherichia coli* L-asparaginase: Prognostic significance and clinical utility of antibody measurement, 8 Am. J. Pediatric Hematol. Oncol. 99 (1986); Howard, J.B. & Carpenter, F.H. (1972) *supra*), which allows an immediate rebound of serum levels of asparagine and glutamine. In an attempt to mitigate both the toxic effects and immunosensitivity associated with the therapeutic utilization of *E. coli* and *E. carotovora* asparaginase, a covalently-modified *E. coli* asparaginase (PEG-asparaginase) was initially developed for use in patients who have developed a delayed-type hypersensitivity to preparations "native" of *E. coli* asparaginase (*see* Gao, S. & Zhao, G., Chemical modification of enzyme molecules to improve their characteristics, 613 *Ann. NY Acad. Sci.* 460 (1990)). However, subsequent studies established that the initial development of an immune response against *E. coli* asparaginase resulted in an 80% cross-reactivity against the PEG-asparaginase with concomitant adverse pharmacokinetic effects—neutralization of PEG-asparaginase activity and normalization of the plasma levels of L-asparagine and L-glutamine (*see* Avramis, V. & Periclou, I., Pharmodynamic studies of PEG-asparaginase (PEG-ASNase) in pediatric ALL leukemia patients, *Seventh International Congress on Anti-Cancer Treatment*, Paris, France (1997)). The development of antibodies directed against *E. coli* (EC) asparaginase and the modified PEG-asparaginase in patients is associated with neutralization of the enzymatic activity of both the EC and

PEG-asparaginases *in vivo,* thus potentially resulting in an adverse clinical prognosis.

II. Effects of Asparaginase Treatment on Spleen and Thymus Histology and Lymphocyte Population.

Examination of the effects of *E. coli* asparaginase treatment on spleen histology and lymphocyte populations shows a marked reduction in both the size and reactivity of the splenic germinal centers, and a concomitant marked reduction in the cytoplasmic immunoglobulin-containing cells (B-cell immunoblasts; *see* Distasio, J. A., *et al.* (1982), *supra*). Additionally, spleen lymphocyte sub-populations show up to a 40% reduction in the percentage of surface immunoglobulin-expressing cells (B-cells) accompanied by an increase in the ratio of Thy-1.2-expressing cells (T-cells), whereas the ratio of Lyt-2 to Lyt-1 cells remains unchanged. In contrast, asparagine deprivation alone, caused by the administration of *W. succinogenes* asparaginase, has no demonstrable effect on spleen histology or lymphocyte marker distribution.

Similarly, histological examination of the thymus following *E. coli* asparaginase administration revealed a pronounced depletion of cortical thymocytes, whereas no changes in thymus histology or cellularity were found after *W. succinogenes* asparaginase administration. Therefore, a comparison of the effects of long-term administration on spleen and thymus histology, cellularity, and weight indicated that *E. coli* asparaginase treatment was associated with a pronounced, sustained reduction in these parameters in both the spleen and thymus.

III. Covalent Modification of Asparaginases and Glutaminases

Many proteins currently used to treat human diseases have extremely short circulating half-lives which limit their efficacy. In addition, the administration of many foreign proteins (including certain recombinant proteins) is associated with allergic hypersensitivity responses which

can also lead to the production of neutralizing antibodies which hasten the rapid elimination of these therapeutic proteins from plasma. To overcome these and other problems, the invention provides a covalent modification procedure to chemically modify proteins, including asparaginases and glutaminases, in order to extend their half-lives, reduce their immunogenicity, and increase their efficacy. This chemical modification regimen involves the systematic alteration of protein structures by conjugating an aliphatic hydrocarbon chain (saturated, partially saturated, or unsaturated, a straight chain, a branched chain, and/or a chain of aromatic) of an acylating agent to polar groups within the protein structure (*see* Figure 7). While this process is generally applicable to any protein to be introduced into a patient, below conditions are described for covalently modifying *E. coli* and *W. succinogenes* asparaginase using an acid chloride.

IV. Compositions, Formulation, and Administration

As described above, asparaginases and glutaminases (and analogs and derivatives thereof) can be used to treat diseases which respond to asparagine or glutamine depletion. These compounds may also be used to treat such diseases prophylactically, or to treat those patients previously diagnosed with and treated for such a disease. For example, a patient previously diagnosed and successfully treated whose disease is otherwise in remission, may experience a relapse. Such patients may also be treated in accordance with the claimed invention.

Asparaginases and glutaminases, and their biologically active analogs and derivatives, can be administered to a patient using standard techniques. Techniques and formulations generally may be found in *Remington's Pharmaceutical Sciences*, 18th ed., Mack Publishing Co., Easton, PA, 1990 (hereby incorporated by reference).

Suitable dosage forms, in part, depend upon the use or the route of entry, for example, oral, transdermal, trans-

mucosal, or by injection (parenteral). Such dosage forms should allow the therapeutic agent to reach a target cell or otherwise have the desired therapeutic effect. For example, pharmaceutical compositions injected into the blood stream preferably are soluble.

Pharmaceutical compositions according to the invention can be formulated as pharmaceutically acceptable salts and complexes thereof. Pharmaceutically acceptable salts are non-toxic salts present in the amounts and concentrations at which they are administered. The preparation of such salts can facilitate pharmaceutical use by altering the physical characteristics of the compound without preventing it from exerting its physiological effect. Useful alterations in physical properties include lowering the melting point to facilitate transmucosal administration and increasing solubility to facilitate administering higher concentrations of the drug. The pharmaceutically acceptable salt of an asparaginase or glutaminase may be present as a complex, as those in the art will appreciate.

Pharmaceutically acceptable salts include acid addition salts such as those containing sulfate, hydrochloride, fumarate, maleate, phosphate, sulfamate, acetate, citrate, lactate, tartrate, methanesulfonate, ethanesulfonate, benzenesulfonate, *p*-toluenesulfonate, cyclohexylsulfamate, and quinate. Pharmaceutically acceptable salts can be obtained from acids, including hydrochloric acid, maleic acid, sulfuric acid, phosphoric acid, sulfamic acid, acetic acid, citric acid, lactic acid, tartaric acid, malonic acid, methanesulfonic acid, ethanesulfonic acid, benzenesulfonic acid, *p*-toluenesulfonic acid, cyclohexylsulfamic acid, fumaric acid, and quinic acid.

Pharmaceutically acceptable salts also include basic addition salts such as those containing benzathine, chloroprocaine, choline, diethanolamine, ethylenediamine, meglumine, procaine, aluminum, calcium, lithium, magnesium, potassium, sodium, ammonium, alkylamine, and zinc, when acidic functional groups, such as carboxylic acid or phenol

are present. For example, see *Remington's Pharmaceutical Sciences, supra*. Such salts can be prepared using the appropriate corresponding bases.

Pharmaceutically acceptable carriers and/or excipients can also be incorporated into a pharmaceutical composition according to the invention to facilitate administration of the particular asparaginase or glutaminase. Examples of carriers suitable for use in the practice of the invention include calcium carbonate, calcium phosphate, various sugars such as lactose, glucose, or sucrose, or types of starch, cellulose derivatives, gelatin, vegetable oils, polyethylene glycols, and physiologically compatible solvents. Examples of physiologically compatible solvents include sterile solutions of water for injection (WFI), saline solution and dextrose.

Pharmaceutical compositions according to the invention can be administered by different routes, including intravenous, intraperitoneal, subcutaneous, intràmuscular, oral, topical (transdermal), or transmucosal administration. For systemic administration, oral administration is preferred. For oral administration, for example, the compounds can be formulated into conventional oral dosage forms such as capsules, tablets, and liquid preparations such as syrups, elixirs, and concentrated drops.

Alternatively, injection (parenteral administration) may be used, *e.g.*, intramuscular, intravenous, intraperitoneal, and subcutaneous injection. For injection, pharmaceutical compositions are formulated in liquid solutions, preferably in physiologically compatible buffers or solutions, such as saline solution, Hank's solution, or Ringer's solution. In addition, the compounds may be formulated in solid form and redissolved or suspended immediately prior to use. For example, lyophilized forms of the asparaginase and glutaminase can be produced.

Systemic administration can also be accomplished by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the

barrier to be permeated are used in the formulation. Such penetrants are well known in the art, and include, for example, for transmucosal administration, bile salts, and fusidic acid derivatives. In addition, detergents may be used to facilitate permeation. Transmucosal administration, for example, may be through nasal sprays, inhalers (for pulmonary delivery), rectal suppositories, or vaginal suppositories. For topical administration, compounds can be formulated into ointments, salves, gels, or creams, as is well known in the art.

The amounts of the active therapeutic agent to be delivered will depend on many factors, including the particular therapeutic agent and the agent's IC_{50}, the EC_{50}, the biological half-life of the compound, as well as the age, size, weight, and physical condition of the patient, and the disease or disorder to be treated. The importance of these and other factors to be considered are well known to those of ordinary skill in the art. Generally, the amount of asparaginase or glutaminase to be administered will range from about 10 International Units per square meter of the surface area of the patient's body (IU/M^2) to 50,000 IU/M^2, with a dosage range of about 1,000 IU/M^2 to about 15,000 IU/M^2 being preferred, and a range of about 6,000 IU/M^2 to about 10,000 IU/M^2 being particularly preferred to treat an auto-immune disease or Graft versus Host Disease. Typically, these dosages are administered via intramuscular or intravenous injection three times per week, *e.g.* Monday, Wednesday, and Friday, during the course of therapy. Of course, other dosages and/or treatment regimens may be employed, as determined by the attending physician.

In addition to administering an asparaginase or glutaminase to treat a disease which responds to asparagine or glutamine depletion, other embodiments of the invention concern administration of a nucleic acid construct encoding the enzyme or an analog thereof. As those in the art will appreciate, a variety of different gene delivery vehicles (GDVs) may be employed for this purpose. GDVs include viral

and non-viral delivery systems. Representative viral delivery systems include recombinant retroviral vectors which provide for stable, long term, and generally low level expression of one or more heterologous genes via integration in the genome of cells transfected by the virus. Here, retroviral GDVs will encode an asparaginase or glutaminase or an analog thereof, and may also include one or more other heterologous genes, for example, a gene encoding a conditionally lethal gene (*e.g.*, thymidine kinase, which converts the pro-drug gancyclovir to its cytotoxic form) to eliminate the transfected cells, if desired.

Other viral delivery systems include those based on adeno-associated virus (AAV) and various alpha viruses, *e.g.*, Sindbis and Venezuelan equine encephalitis virus. These other viral GDVs may provide for higher level expression, or expression for different duration, of the desired heterologous gene(s). As those in the art will appreciate, the host range for the particular virus employed may be altered by techniques well known in the art.

Non-viral GDVs useful in the practice of these embodiments of the invention include, among others, so-called "naked DNA" systems which provide the desired heterologous gene(s) in functional association with an appropriate promoter (which in certain embodiments may be an inducible or tissue-specific promoter) encoded by the nucleic acid construct. Other regulatory elements may also be included, for example, enhancers and other activators of gene expression. Preferably, such non-viral systems are incorporated into liposomes or are associated with polycationic reagents to facilitate introduction of the nucleic acid construct into cells of the patient. Of course, other components can also be included in such GDVs, *e.g.*, molecules to target one or more particular cell types, fusogenic peptides to facilitate endocytotic vesicle escape, *etc.* Construction of these and other GDVs useful in the practice of this invention are within the skill of those in the art.

Detailed Description Of The Preferred Embodiments

The following examples will serve to further illustrate various aspects of the present invention and are not intended to act in any manner as limitations on the claimed invention. In addition, methodologies are provided which will permit one of ordinary skill within the relevant arts to determine whether a derivative asparaginase or glutaminase is appropriate for utilization in the clinical therapeutic treatment of humans. For a discussion of molecular biology techniques which can be used in the practice of this invention, in addition to those described below, *see Molecular Cloning, A Laboratory Manual*, 2d ed., ed. Sambrook, *et al.*, Cold Spring Harbor Laboratory Press, 1989, and *Current Protocols In Molecular Biology*, ed. Ausubel, *et al.*, John Wiley & Sons, Inc., 1995.

Example 1: In Vitro Culture of W. succinogenes

W. succinogenes was grown in 10-15 liters of liquid culture media containing 0.4% yeast extract, 100 mM ammonium formate, and 120 mM sodium fumerate. The medium was adjusted to pH 7.2 prior to autoclaving. After autoclaving, a 0.2 μm filter-sterilized solution of thioglycolate was added to the room temperature culture medium to give a final concentration of 0.05%. The cultures were incubated with continuous agitation on a shaking platform in a 37°C warm-room. For large scale culture, a 500 mL pre-culture was utilized to inoculate 10-15 liters of complete culture medium.

The bacteria were collected after the cultures had reached a optical density of approximately 1.1 at a 650 nm wavelength, by centrifugation using a Sorvall high-speed continuous flow rotor. Following centrifugation, the cells were washed in a buffer containing 0.15 M sodium chloride, 0.1 M magnesium chloride, and 0.01 M mercaptoethanol. The cells were then resuspended in 0.1 M borate buffer (pH 9.0) at a final concentration of 0.5 g wet cell weight/mL borate

buffer and stored frozen until subsequent processing for enzyme purification.

Example 2: Animals and Cell Lines

The murine model animals utilized in these experiments were Balb/C or C3H mice of 9 to 12 weeks in age (Jackson Laboratories, Bar Harbor, ME).

The therapeutic activity of L-asparaginases was determined utilizing the 6C3HED Gardner's lymphosarcoma (Gardner, W.U., *Cancer Res., vol.* 4: 73 (1944)) and P1798 lymphosarcoma cell lines (ATCC) which as ascites tumors in C3H and Balb/cc mice, respectively. Alternately, the two lymphosarcoma cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. The 6C3HED Gardner's lymphosarcoma originated in the thymus of C3H mice that were initially given high doses of estradiol. The lymphosarcoma was subsequently perpetuated by serial transplantation in the C3H mice.

W.S. asparaginase showed potent anti-tumor activity.

Example 3: Isolation of *W. succinogenes* Genomic DNA

Genomic DNA from *W. succinogenes* was extracted from bacteria grown in basal medium. Typically, bacterial cells from a 50 mL of culture were collected by centrifugation and resuspended by gentle vortexing in 1.5 mL TE buffer (pH 7.0). To the cell suspension was added 15 µL of 10% SDS to give a final concentration of 0.1% and 3 µL of a 20 mg/mL stock solution of proteinase K. The mixture was then incubated at 37°C for approximately 60 minutes, followed by several phenol/chloroform extractions. The genomic DNA was ethanol precipitated and collected by centrifugation. The *W. succinogenes* genomic DNA so isolated was sufficiently pure to use in high stringency PCR amplification.

Example 4: PCR Amplification of *W. succinogenes* Asparaginase Sequences

The nucleotide sequence of a 2.5 kb Hind III fragment containing the 993 nucleotide coding region of *W. succinogenes* asparaginase was published in 1995. *See* GenBank accession number X89215. The elucidation of this sequence facilitated the synthesis of primers specific for PCR amplification of the gene coding, for the *W. succinogenes* enzyme. As illustrated in Figure 1, the forward and reverse *W. succinogenes* asparaginase-specific PCR primers forward and reverse had the following sequences:

5' -TCCGGATCCAGCGCCTCTGTTTTGATGGCT-3'	Forward PCR Primer [SEQ ID NO. 1] (BamHI] Restriction Site Underlined)
5' -TGGGAATTCGGTGGAGAAGATCTTTTGGAT-3'	Reverse PCR Primer [SEQ ID NO. 2] (EcoR1 Site Restriction Underlined)

It should be noted that the genomic *W. succinogenes* asparaginase coding sequence does not naturally contain either a BamH1 or EcoR1 restriction site. However, PCR amplification utilizing these aforementioned primers introduced a BamH1 and EcoR1 restriction site to the 5'- and 3'-termini, respectively to facilitate directional cloning of this amplified genomic sequence into sequencing and/or expression vectors.

With respect to PCR amplification, *W. succinogenes* genomic DNA (purified as per Example 3) was subjected to 30 cycles of PCR amplification under the following reaction conditions: 10 µL PCR II reaction buffer; 6 µL of 25 mg/mL magnesium chloride, 8 µL of 10 mM stock solutions of dNTPs, 1 µL of Taq DNA polymerase (Stratagene Corp.); 1 µL (about 50 ng) each of the *W. succinogenes* asparaginase-specific

forward and reverse PCR primers; 1 µL of *W. succinogenes* genomic DNA; and nuclease-free PCR-grade water to bring the reaction mixture to 100 µL total volume. Following amplification, 2 µL of the PCR products were electrophoresed through a 1% agarose gel and stained with propidium iodine to assess both the specificity of the amplification reaction and the molecular weight of the resulting DNA fragments. The amplification resulted in the production of a homogeneous, 1.0 kb *W. succinogenes* asparaginase-specific DNA fragment.

Example 5: Cloning of *W. succinogenes* Asparaginase Sequences

The amplified *W. succinogenes* asparaginase-specific amplified DNA fragment was subsequently sub-cloned into the BamH1 and EcoR1 sites of the PCRII cloning vector (Stratagene, La Jolla, CA) utilizing the following reaction conditions: 2 µL of the PCR amplified reaction products, 2 µL of the PCRII cloning vector; 1 µL of 10X ligation buffer; 4 µL of T_4 DNA ligase (Stratagene, La Jolla, CA); and distilled/deionized water to bring the total reaction volume to 10 µL. The ligation reaction was incubated at 16°C overnight and 2 µL of this reaction was utilized to transform competent *E. coli* strains DH-5α and M15.

IPTG-induced colorimetric selection (medicated by expression of β-galactosidase in the presence of X-GAL) was utilized to identify recombinant bacterial colonies. Three white colonies (putative positive recombinants) and one blue colony (putative negative recombinants) were chosen, inoculated into a 5 mL culture of LB medium containing 100 µg/mL ampicillin, and incubated overnight at 37°C on a shaking platform. Plasmid DNA was isolated from these cultures via standard DNA "mini-prep" methodology and the DNA was dissolved in 30 µL TE buffer and digested with 3 different restriction endonucleases: BamH1; EcoR1; and BamH1/EcoR1, to ensure that the isolated plasmid DNA

contained the expected 1.0 kb *W. succinogenes* asparaginase-specific insert.

The electrophoretic results, as illustrated in Figure 3, lanes 2 and 4, demonstrated that colonies #1 and #3 contained the expected 1.0 kb insert. To confirm that these clones contained the *W. succinogenes* asparaginase gene, the *W. succinogenes* asparaginase-specific PCR primers were used to amplify the *W. succinogenes* asparaginase-specific fragments isolated from the aforementioned clones (Figure 3, lanes 2 and 4). These primers did not mediate amplification of non-insert-containing bacterial DNA (Figure 3, lane 3). Results of this second PCR amplification demonstrated that colonies #1 and #3 contained the *W. succinogenes* asparaginase-specific DNA insert within the PCRII cloning vector, resulting in the generation of a 1.0 kb amplification product (see Figure 3, lanes 2 and 4).

The *W. succinogenes* asparaginase-specific DNA insert in the PCR II cloning vector was then removed by BamH1 and EcoR1 digestion of 10 g of plasmid DNA derived from colony #1, gel-purified via the use of Gene Clean Kit® (Stratagene, La Jolla, CA). The DNA insert was eluted from the gel with 10 µL distilled/deionized water and then ligated overnight at 16°C into the similarly restricted pGEX-2T (Amersham Pharmacia Biotech, Piscataway, N.J.) and pET-28a (Novagen, Inc., Madison, WI) vectors under the following reaction conditions: 3 µL DNA insert; 3 µL vector DNA; 4 µL 5X ligation reaction buffer; 1 µL T_4 DNA ligase; and 9 µL of distilled/deionized water to give a final reaction volume of 20 µL. 10 µL of each ligation reaction mixture was used to transform 50 µL of competent *E. coli* DH-5α cells. Transformants were then plated onto LB agar plates containing 100 mg/mL ampicillin. Positive transformants (*i.e., W. succinogenes* asparaginase-specific DNA insert-containing transformants, pGEX-2T-WSA and pET-28-WSA, respectively) were obtained following approximately 18 hours of incubation at 37°C. To confirm that the transformants contained the *W. succinogenes* asparaginase-specific DNA

insert, restriction endonuclease digestion using BamH1 and EcoR1 was performed, as well as PCR amplification and DNA sequence analysis. Results of these analyses demonstrated that each of the selected "positive" transformants contained the *W. succinogenes* asparaginase-specific DNA insert. The nucleotide sequence of the *W. succinogenes* asparaginase-specific DNA insert is shown in Figure 6 [SEQ ID NO. 3], which sequence contains 117 nucleotides 5' to the initial codes of the Wolinella gene and 23 nucleotides 3' to the gene's termination codon.

Example 6: Expression of Recombinant *W. succinogenes* Asparaginase Analogs

To facilitate isolation of the recombinant *W. succinogenes* (rWS) asparaginase protein, several types of epitope-labeled asparaginase analogs have been constructed. These epitope labels included: influenza hemagglutinin (HA); glutathione-S-transferase (GST); DYLD (FLAG); and poly-histidine (p-His). In each instance, the label is placed on the N-terminus of the enzyme.

The following methodologies are utilized to isolate these various epitope labeled rWS asparaginase proteins:

(1) GST-sepharose (Pharmacia AB, Upsala, Sweden) column chromatography is utilized to purify the GST-labeled rWS asparaginase enzyme expressed from the pGEX-2T-WSA vector, followed by cleavage by thrombin.

(2) Protein-G-sepharose immobilized anti-HA and anti-FLAG antibodies (Pharmacia AB, Upsala, Sweden) is utilized to affinity purify the HA-or FLAG-labeled rWS asparaginase enzyme.

(3) Nickel resin (Ni-NTA [nitilo-tri-acetic acid resin]; Novagen, Inc., Chatsworth, CA) is used to affinity purify p-His-labeled rWS asparaginase enzyme.

More specifically, for example, production of poly-histidine (p-His)-labeled, glutathione-S-transferase (GST)-rWS asparaginase requires the induction of positively transformed *E. coli* with IPTG, followed by harvesting of the bacteria (see Hochuli, E., & Dobell, N, New metal chelate absorbents selective for protein and peptide containing neighboring histidine residues, 411 *J. Chromatography* 177 (1987)). In such expression systems, vectors such as pGEX-2T and pET-28a expression vectors may be utilized to facilitate the expression of a non-epitope-labeled form of the rWS asparaginase following IPTG induction. The p-His-labeled constructs, localized in the N-terminus of the rWS asparaginase, can then be sub-cloned into the BamHl to EcoRl site of the pET-28a vector (Novagen, Inc., Chatsworth, CA) for expression of the p-His-labeled rWS enzyme.

Example 7: Purification of Native *Wolinella succinogenes* Asparaginase

The native, homotetrameric form of *W. succinogenes* asparaginase was purified according to the following methodology. *W. succinogenes* cell lysates were prepared by subjecting bacteria cultured and frozen in accordance with Example 1 to 3 to 4 freeze/thaw cycles with sonication, followed by high-speed centrifugation to remove cell debris. After centrifugation, the supernatant was brought to 0.1 M concentration of ammonium sulfate at a temperature of 4°C. The mixture was then brought to a final volume of 120% by the addition of a 2% protamine solution, followed by centrifugation for 30 min. at 21,000 x g. The supernatants were recovered, pooled, and brought to a 50% ammonium sulfate saturation and equilibrated for 30 minutes on ice with continuous stirring. The resulting solution was then dialyzed against 0.01 M potassium phosphate buffer (pH 8.0) and applied to a 3 cm x 20 cm hydroxyapatite column (prepared by: Pharmacia, Inc.) equilibrated with 0.1 M potassium phosphate buffer pH 8.0.

The *W. succinogenes* asparaginase was eluted from the hydroxyapatite column utilizing step-wise concentrations of phosphate buffer (*i.e.*, 0.10, 0.20, 0.25, 0.30, 0.35 M phosphate buffer, pH 8.0). The eluted fractions (10 mL/fraction) were collected, assayed for asparaginase enzymatic activity, and pooled. The enzymatically-active fractions were dialyzed against 0.1 M sodium borate buffer (pH 7.0) and applied to a 3 cm x 20 cm DEAE-Sephadex column (prepared by Pharmacia, Inc.) equilibrated in 0.1 M sodium borate buffer, pH 7.0. The enzyme was eluted by use of a linear gradient of sodium chloride (0 to 1.0 M) in 0.1 M sodium borate buffer (pH 7.0). 60 mL asparaginase-containing fractions were retained. *W. succinogenes* L-asparaginase prepared utilizing this methodology has been shown to be homogeneous by SDS-PAGE electrophoresis and silver staining.

E. coli EC-2 asparaginase (Merck, Sharp & Dohme, West Point, PA) was further purified by gel filtration on Ultragel® AcA-44 (LKB Instruments, Inc., Rockville, NM). *Erwinia carotovora* asparaginase (Microbiological Research Establishment, Salisbury, England) was provided by Pharmaceutical Resources Branch of the National Cancer Institute.

Example 8: Determination of the Biochemical Characteristics of Asparaginase

The X-ray crystallographic structures of several microbial asparaginases have been elucidated (*see* Lubkowski, J. & Palm, N. (1996), *supra*). Recombinant *W. succinogenes* asparaginase which possesses acceptable clinical properties has the following characteristics: (1) catalytic activity *in vitro,* (2) preferably a native-protein-like homotetrameric structure required for functional enzymatic catalysis, and (3) with respect to the recombinant form of *W. succinogenes* asparaginase, similar to that of the native, homotetrameric form of *W. succinogenes* asparaginase, greater substrate specificity for L-asparagine and not catalyzing the

deamidation of L-glutamine to any physiologically significant degree.

In order to quantitate the biochemical characteristics of both the native, homotetrameric and recombinant asparaginase enzymes, K_m and V_{max} enzyme kinetics, substrate specificity, pH optimum, and temperature optimum can be determined. In addition, SDS-PAGE under both reducing and non-reducing conditions, followed by silver and Coomassie Blue staining of the gels, can be utilized to establish enzyme homogeneity, evaluate subunit composition, and determine enzyme molecular weight (*see* Park, R. & Liu, K., A role for Shc, grb2 and raf-1 in FcR1 signal relay, 271. *J. Biol. Chem.* 13342 (1996).

The enzymatic activity of L-asparaginase can be quantitatively determined by the amount of ammonia produced upon the hydrolysis of 0.08 M L-asparagine using 0.01 M sodium phosphate buffer (pH 7.0) as the reaction buffer (*see* Durden, D. L. & Distasio, J. A. (1980), *supra*). The assay mixture can consist of 10 to 40 IU of a homogeneous solution of L-asparaginase enzyme diluted to 2.0 mL with 0.01 M sodium phosphate buffer (pH 7.0). Briefly, this assay system measures the deamidation of L-asparagine indirectly by quantitating the release of NH_3 as colormetrically-detected by Nessler's Reagent. A standard curve of NH_4OH may be prepared to initially derive an extinction coefficient for NH_3, based upon absorbance at 420 nm. The enzyme reaction may be initiated by the addition of the L-asparagine substrate (0.04 M). For the determination of K_m and V_{max} enzyme kinetics, a more sensitive NADPH-dependent L-asparaginase assay system can utilized (*see* Distasio, J. A. & Niederman, T. (1976), *supra*).

Example 9: Therapeutic Administration of Asparaginase in Murine Animal Models

The recombinant and native forms of *W. succinogenes* asparaginase may be titrated between 5 and 50 IU per

injection and the mice can receive up to 3 daily intraperitoneal (I.P.) injections at each dose. Toxicological and pharmacological studies for the native and recombinant enzymes can be performed by the determination of serum enzyme activity (*i.e.*, serum enzyme half-life) as described in Example 8.

Example 10: Determination of Asparaginase Enzymatic Activity (Serum Half-Life)

Serum half-life determinations can be performed on Balb/c mice intraperitoneally-injected with 5 or 10 IU of native (WS) or recombinant (rWS) *Wolinelia succinogenes* asparaginase. Enzyme half-life measurements can be performed by a slight modification of a previously published procedure (*see* Durden, D. L., *et al.*, Kinetic analysis of hepatotoxicity associated with anti-neoplastic asparaginases, 43 *Cancer Res.* 1602 (1983)). Specifically, enzyme half-life measurements can be performed by obtaining a 5 µL blood sample from the tall vein of the Balb/c mice at specific intervals following the I.P. injection of the WS or rWS asparaginase. The blood samples are then kept on ice until all samples had been collected. Once sampling was completed, each 5 µL blood sample can then be immediately pipetted into 0.5 mL of cold 1.19% sodium chloride in 0.1 M sodium phosphate buffer (pH 7.0) and mixed by vigorous vortexing.

To determine serum asparaginase activity (and hence serum half-life), two 0.2 mL aliquots from each time point can be equilibrated in a 37°C water bath. The enzymatic reaction is subsequently initiated by the addition of 0.03 mL of 0.04 M L-asparagine, pre-equilibrated to 37°C prior to addition, into one of the 0.2 mL samples. The other 0.2 mL aliquot receives only 0.3 mL of distilled water and will serve as a control "blank." The substrate-containing reaction tube may be incubated at 37°C for 1 hour after which the reaction is stopped by the addition of 0.2 mL of 5% TCA.

In addition, a 0.2 mL aliquot of 5% TCA is also added to the control "blank." The tubes are then centrifuged at 5000 x g to remove the resulting TCA-produced precipitate. Enzymatic activity may be colormetrically-determined by the addition of a 0.2 mL aliquot of the substrate-containing sample to 0.2 mL of distilled water and 0.2 mL a freshly-prepared Nessler's Reagent and the absorbance at 420 nm is read using a spectrophotometer (Gilford Instrument Laboratories, Oberlin, OH).

Example 11: Determination of the Anti-Neoplastic Activity of Asparaginase

The anti-neoplastic (anti-lymphoma) activity of homogeneous preparation of both native (WS) and recombinant (rWS) *W. succinogenes* asparaginase, as well as that of native *E. coli* (EC) and *E. carotovora* (Erw) asparaginases, can be determined utilizing the 6C3HED Gardner lymphosarcoma cell line implanted in C3H mice. This lymphoid tumor originated in the thymus of C3H mice given high doses of estradiol and was perpetuated by serial transplantation in the C3H mice. In these studies, the tumor is maintained as an ascites tumor through I. P injection of 2 x 10^8 viable lymphosarcoma cells in 0.1 mL of PBS (pH 7.0).

To determine asparaginase anti-tumor activity, 2.5 x 10^6 viable 6C3HED lymphosarcoma cells from an ascites tumor is injected in a volume of 0.05 mL of PBS (pH 7.0) subcutaneously in the left ventral groin of 9 to 12 week-old C3H mice. Similarly, in another series of experiments, 2.5 x 10^6 viable P1798 lymphosarcoma cells from an ascites tumor is injected in a volume of 0.05 mL of PBS (pH 7.0) subcutaneously in the left ventral groin of 9 to 12 week-old Balb/c mice (*see* Jack, G. W., et al., The effect of histidine ammonia-lyase on some murine tumors, 7 *Leukemia Res.* 421 (1983)). Palpable solid tumor growth generally occurred within 4 to 7 days after injection of the lymphosarcoma cells. Changes in solid tumor volume are then

subsequently measured by daily caliper-based measurement of tumor dimensions along three axes. When the average tumor volume reaches 1 cm^3, intraperitoneal injection of asparaginase can be performed. A total dosage of 3 or 6 IU of asparaginase may be administered in a total of six I. P injections of 0.5 or 1.0 IU asparaginase/injection, respectively. Injections may be administered twice daily for three consecutive days.

The negative control animal group receives I.P. injections of 0.01 M phosphate buffer (pH 7.0) utilizing a similar injection schedule. *E. coli* and *E. carotovora* asparaginases serve as positive controls for comparison of anti-tumor activity in this series of experiments. Student's t-test will be utilized for all statistical analysis of data.

Example 12: Immune Cross-Reactivity *W. succinogenes* Asparaginase

This example describes how it was determined if antibodies in patients known to neutralize *E. coli* asparaginase react with *W. succinogenes*. Specifically, an ELISA assay was performed to make this determination, as described below.

The ELISA assay was performed on two 96 well microtiter plates, as follows: asparaginase (EC on one plate, WS on the other) was diluted in carbonate buffer (prepared by dissolving 1.59 g Na_2CO_3, 2.93 g $NaHCO_3$, and 0.2 g NaN_3 in 1 L of purified water; pH was adjusted to 9.0 - 9.5 using 1N HCl or 1N NaOH; the buffer was stored at 4°C for no more than two weeks before use) to a final concentration of 0.10 IU/mL. 54 wells on each plate were coated with 100 µL of the respective diluted asparaginase solution and incubated overnight at 4°C after being wrapped in aluminum foil to allow the enzyme to become associated with the plates.

The following morning the plates were removed, and the solution from each of the wells was removed. These wells

were then blocked with 300 µL of a 1 mg/mL solution of BSA-PBS blocking buffer, pH 7.0 (prepared fresh by adding the appropriate amount of bovine serum albumin to PBS buffer, 0.010 M sodium phosphate, pH 7.0 - 7.2, 0.9% saline). The plates were then incubated for 1 hour at room temperature. Thereafter, the plates were washed with 300 mL of saline-Tween buffer (0.145 M NaCl, 0.05% Tween 20) per well using a Dynatech Ultrawash plate washer.

The antibodies used to screen the two plates were diluted as follows: 1:100, 1:1,000; 1:2,000; 1:4,000; 1:8,000; 1:16,000; and 1:32,000. As a control, serum from a normal human patient was used. Patient serum and rabbit anti-EC asparaginase serum and normal human serum were diluted in PBS-Tween (PBS containing 0.05% Tween 20) and 100µL of each dilution was placed on each plate in triplicate according to the following grid:

CONTROL			HUMAN PATIENT			RABBIT ANTIBODIES		
1	2	3	1	2	3	1	2	3
1:1,000	1:1,000	1:1,000	1:1,000	1:1,000	1:1,000	1:1,000	1:1,000	1:1,000
1:2,000	1:2,000	1:2,000	1:2,000	1:2,000	1:2,000	1:2,000	1:2,000	1:2,000
1:3,000	1:3,000	1:3,000	1:3,000	1:3,000	1:3,000	1:3,000	1:3,000	1:3,000
1:4,000	1:4,000	1:4,000	1:4,000	1:4,000	1:4,000	1:4,000	1:4,000	1:4,000
1:8,000	1:8,000	1:8,000	1:8,000	1:8,000	1:8,000	1:8,000	1:8,000	1:8,000
1:16,000	1:16,000	1:16,000	1:16,000	1:16,000	1:16,000	1:16,000	1:16,000	1:16,000
1:32,000	1:32,000	1:32,000	1:32,000	1:32,000	1:32,000	1:32,000	1:32,000	1:32,000

After adding the above dilutions, the plates were incubated for at least 1.5 hour at room temperature, followed by washing each plate three times with saline-Tween as described above. A 1:1,000 dilution of Horse radish peroxidase-conjugated goat anti-human immunoglobulin (BioSource International) was then prepared in PBS-Tween. 100 µL of the HP-conjugated anti-human Ig was then added to each well. The plates were then covered and allowed to incubate at room temperature for 1 hour.

After the 1 hour incubating each plate was again washed three times with saline-Tween, as before. To detect antibody binding, 100 µL of OPD (o-phenylenediaminedihydrochloride) substrate (40 mg of OPD in 100 mL a citrate phosphate buffer (0.1M, pH 6.0, prepared by combining a solution containing 13.4 g $Na_2HPO_4 \cdot 7H_2O$ (dibasic) in 500 mL distilled water with an amount of a solution containing 9.60 g citric acid (anhydrous) in 500 mL distilled water sufficient to adjust the pH to 6.0) with 334 µL of 3% H_2O_2 prepared immediately before use and kept at room temperature in the dark) was added to each well and allowed to incubate at room temperature in the dark for approximately 40 minutes. The reaction in each well was stopped by adding 100 µL of 1 M phosphoric acid. The absorbance of each well was then measured at 40 nm.

As is shown in Figure 8, high titer neutralizing antibodies against the *E. coli* enzyme present in patient plasma failed to bind to the *Wolinella* asparaginase. This figure shows one of 6 plasma specimens collected from patients known to be allergic to the *E. coli* enzyme as well as rabbit antisera raised against the E. coli asparaginase. None of these anti-*E. coli* reactive antisera bind or neutralize the *Wolinella* asparaginase activity (Figures 8 and 9). From these data it was concluded that the *W. succinogenes* enzyme is immunologically distinct from *E. coli*, and that the *Wolinella* enzyme can be used in patients allergic to the *E. coli* enzyme (as exemplified by titration

of patient plasma shown in Figure 8 and rabbit anti-*E. coli* antisera shown in Figure 9).

A highly specific antisera against the *W. succinogenes* enzyme which does not cross react with *E. coli* asparaginase in Western blot analysis has also been prepared. This reagent is useful for performing immunological characterizations of the native, recombinant, and various analog forms of the *Wolinella* enzyme. Analysis of native, recombinant, and analog forms of *W. succinogenes* asparaginase for this type of immunologic cross reactivity will be useful in characterization of genetically and chemically modified proteins. Importantly, these analyses will be applied to analysis of clinical specimens during phase I and II clinical trials of the different forms of the *W. succinogenes* enzyme.

Example 13: Methodology for Protein Modification using Acylation.

Protein acylation is accomplished by using different acylating agents, such as acyl halides (*e.g.*, acyl chlorides), carbodiimide compounds, or acid anhydrides, each with a different number of carbon atoms comprising a straight or branched aliphatic chain attached to the carbonyl, or the modified carbonyl (in the case of carbodiimides), carbon atom. The acylating agents contemplated for use in practicing this invention have the ability to react with a polar group contained within the peptide sequence of a protein to form an amide side chain. The polar group is the side chain of any of the amino acids in the primary sequence, for example, the amine group of lysine or arginine, the hydroxy group of threonine, serine, or tyrosine, or the thiol group of cysteine. Preferably, the reaction is carried out under conditions which do not substantially reduce (*i.e.*, reduce by more than 90%, preferably less than 50%, and more preferably less than 25%) the catalytic activity of the enzyme.

Briefly, the chemical reaction was started at zero time with the dropwise addition of acetyl chloride to 5,000 IU of asparaginase, derived from either *E. coli* or *W. succinogenes*, in a volume of 10 mL of 0.1 M borate buffer at pH 8.5. The final concentration of each acid chloride is 0.1 M. The chemical reaction involves a nucleophilic attack of the polar group, *e.g.*, the free amino group, within the peptide sequence of the protein, *e.g.*, asparaginase (which is maintained in an unprotonated form in the borate buffer, pH 8.5) with the reactive acylating agent. The polar group reacts with the acylating agent yielding an aliphatic hydrocarbon modified amino acid side chain. If the acylating agent is an acyl halide, an equivalent of the respective hydrohalic acid is produced. Thus, if the acylating agent is acyl chloride and the amino acid to be modified is lysine, then the reaction yields an acylated amino group and 1 equivalent of HCl (*see* Figure 7). To prevent acid conditions from destroying the structure of the protein molecule (decreasing yield of enzyme, Table 1, below), a 1 N solution of NaOH is added drop-wise to the reaction mixture every 5-10 seconds. Aliquots of 2 mL were removed at the indicated reaction times (*see* Table 1, below), and immediately dialyzed against 0.01 M phosphate buffer at pH 7.0. Protein concentration is measured by Bradford method. Enzyme activity is determined by the amount of ammonia produced upon hydrolysis of L-asparagine (0.08 M L-asparagine) with a Nessler's reagent (see Durden, D.L. et al, *Cancer Res.* 40: 1125, (1980)). Free amino groups are measured by the method of Habeeb (*see* Habeeb, A.F.S.A., *Analytical Biochemistry*, 14:328, 1966).

TABLE I

Effect of acylation with acetyl chloride on *W. succinogenes* asparaginase

	Reaction time[a] (hr)	Specific Activity[b,c] (IU/mg)	Reduction of free amines[d] (%)	Recovery of Activity[c] (%)	Half-Life (hr)
Native enzyme	0	150.0	0	100.0	1.8
Derivatized enzyme	0.5	120.0	29.0	80.0	8.0
	1.0	129.0	26.8	86.0	8.2
	2.0	130.0	32.4	86.6	7.4
	3.0	120.0	30.2	80.0	7.3
	4.5	90.0	31.3	60.0	6.2

a. The reaction is started at time 0 with the addition of acetyl chloride to 5,000 IU of *W. succinogenes* asparaginase in 10 mL of 0.1 M borate buffer, pH 8.5. Aliquots of 2.0 mL are removed at the times indicated and dialyzed against 0.01 M phosphate buffer, pH 7.0.

b. Protein is measured in triplicate by method of Bradford.

c. Enzyme activity is measured by determining the amount of ammonia produced upon hydrolysis of L-asparagine with Nessler's reagent.

d. Free amino groups are measured by method of Habeeb.

Acyl modification is performed with acylating agents of different aliphatic chain lengths, e.g., a 2 carbon aliphatic chain (C2), a 4 carbon aliphatic chain (C4), a 6 carbon aliphatic chain (C6), etc. Importantly, each specific protein (e.g., asparaginase) has different numbers of free polar groups in different positions within the

protein molecule and hence each protein is optimally modified with a different length acylating agent which conjugates a different aliphatic carbon chain to the free amino groups. These include, for example, acetyl chloride (C2), butyryl chloride (C4), hexanoyl chloride (C6), decanoyl chloride (C10), as well as the use of branched chain acid chlorides including trimethyl-acetyl chloride. Also, different acylating agents may be used for different proteins. For example, with some proteins acetyl chloride may be used, whereas for other proteins acetic anhydride may be the best acylating agenst. By way of illustration, the covalent modification of the *W. succinogenes* asparaginase with the acetyl chloride is presented in Table 1.

A. Results of Modification

There are a number of problems that have been associated with the use of enzymes for therapeutic purposes. Many of these enzymes have extremely short half-lives which severely limits their effectiveness *in vivo*. The modification of proteins using organic modification techniques of the present invention is a promising solution to many of these problems. The C2 modification of *W. succinogenes* asparaginase results in an enzyme which has a half-life of 8.2 hours in mice as compared to the 1.8 hour half-life of the native enzyme. The increase in half-life is consistent with the time course of acetylation reaction (resulting in 20-40% decrease in enzyme activity while the activity of the *W. succinogenes* asparaginase decreases with the increasing reaction time). An about 80% recovery of enzyme activity after a 30 min. reaction time was observed, a time of maximum alteration of pharmacokinetic extension of half-life to 8.0 hours. Other modification procedures which involve polymerization (*e.g.*, polyethylene-glycol modification) result in heterogenous groups of modified reaction products which may not be suitable for

administration in humans. The acid chloride modification procedure is a systematic approach which does not yield such heterogeneity in reaction products (*see* Figure 7). The greater reproducibility and more restricted nature of reaction products result in a well controlled modification of proteins and a more reliable product with predictable extension of half-life which decrease the immunogenicity, and with the advantage of being able to very carefully control the extent of modification of the polar groups present in a specific protein molecule. Current data modifying *W. succinogenes* asparaginase demonstrate that the enzyme is modified with a C2 acylation reaction which results in the augmentation of half-life approximately four fold. The modification of the free amino groups and the asparaginase molecule is responsible for extension of half-life. It is suggested that the extension of half-life will correlate with a decrease in the electrostatic charge, increase in hydrophobicity and decreased immunogenicity of the *Wolinella* enzyme. The extension of half-life and decreased immunogenicity will increase the efficacy of the *W. succinogenes* enzyme when this drug is used in the treatment of acute lymphoblastic leukemia, autoimmune disease, or AIDS, for example, in humans. Through this modification procedure, we are able to generate foreign proteins which have lower immunogenicity, extended half-life, and augmented efficacy. With this systematic approach of modification, any protein can be modified and the modified protein can then be used in the treatment of human disease. Essentially, any protein that has polar groups available in its native state (essentially all known proteins) is amenable to the modification technique of the present invention. Hence this invention extends to all proteins currently used in treatment of human, animal and plant diseases.

Example 14: Mouse Autoimmune Disease Model

Collagen induced arthritis (CIA) in DBA/1 mice is a recognized experimental autoimmune disease model that reflects aspects of human rheumatoid arthritis. When immunized with human collagen type II, these mice develop severe arthritis with inflammation and erosions of their joints. Cellular and humoral immune mechanisms against collagen characterized by synovial proliferation and joint infiltration by inflammatory cells are believed to be involved in the pathogenesis of this arthritis model.

Susceptibility to CIA is linked to HLA class II but also requires the presence of T cells expressing variable V beta chains of their T cell receptor. Due to the T cell depleting effect of L-Asparaginase, the severity of CIA can be reduced and arthritis can be prevented (or, if initiated, the progression of the disease at least halted) by prophylactic administration of L-Asparaginase prior to immunization with collagen.

DBA/1 (H-2q) mice were purchased from Jackson Laboratories (Bar Harbour, ME), and males 8-12 weeks of age were used for immunization experiments.

A. Induction of Arthritis

Sedated mice were immunized with 200 µg of bovine collagen type II emulsified 1:1 in complete Freud's adjuvant (CFA) (Difco, Detroit, MI) at the base of the tail. Arthritis typically developed 4-6 weeks after immunization in 60-80% of the animals. All animal manipulations were performed under ether anesthesia.

B. Assessment of Arthritis

Arthritis of fore and hind paws was assessed using a subjective scoring system in which "0" = normal, "1" = minor swelling or erythema, "2" = pronounced, edematous swelling, and "3" = rigidity. Each limb was graded separately, giving a maximal possible score of 12 per mouse.

C. Effect of L-Asparaginase on Existing Arthritis (therapeutic protocol)

At onset of arthritis symptoms, mice were treated with 5, 10, 25, or 50 IU, respectively, of EC asparaginase intraperitoneally once a day for a total of 3 months and compared to untreated controls. Additional experiments using EC-PEG and WS asparaginases can be similarly conducted using the same outcome parameters. WS asparaginase, which is believed to solely deplete L-asparagine, has no known immunosuppressive effects. Thus, the effect of L-asparagine depletion on the severity and prevention of arthritis can be assessed using the WS enzyme.

Arthritis was scored every other day for the first month, every third day during the second month, and once a week in the third month after onset and treatment of arthritis symptoms. After 3 months, mice were sacrificed for histopathological studies.

The data showed that *E. coli* asparaginase has potent anti-arthritic activity. *E. coli* asparaginase treatment resulted in the reversal of pre-existing arthritis in this model (*see* Figures 10 and 11). Given the recognized correlation between this model and human disease, asparaginase treatment should reverse, prevent, or halt the progression of human rheumatoid arthritis and other autoimmune states.

Other data showed that *E. coli* asparaginase treatment reversed the arthritic state induced by collagen and LPS *see* Figure 12). Activity in this highly resistant form of autoimmune arthritis confirmed the results from the mouse model shown herein, and further supports the usefulness of asparaginases and glutaminases in the treatment of autoimmune diseases. The differences in arthritic scores between *E. coli* treated animals and control animals were statistically significant ($p<0.001$).

D. Effect of L-Asparaginase on Arthritis Prevention (preventive protocol)

To study the ability of L-Asparaginase to prevent arthritis, DBA/1 mice were treated I.P. with 5, 10, 25, or 50 IU, respectively, of EC asparaginase prior to immunization (-1), parallel to immunization (0), and then at the consecutive days 5, 10, 15, and 30 thereafter. Arthritis was scored every other day for the first month, every third day during the second month, and once a week in the third and fourth month after onset of arthritis symptoms.

After four months mice were sacrificed for histopathological studies. The administration of *E. coli* asparaginase concomitantly with type II collagen in the DBA mouse model completely abrogated the development of autoimmune CIA. These results also strongly support the role for asparaginase and/or glutaminase in the prevention and/or treatment of autoimmune and/or Graft Versus Host disease in humans.

E. Assessment of Histology

Removed limbs were fixed in 10% buffered formaldehyde for four days. After decalcification using 5% formic acid, specimens were embedded in paraffin, cut into thin slices, and stained for hematoxylin and eosin. Sections were obtained from the femoro-patellar area for the knee joints and calcaneal area for the ankle joints. Histological parameters included the amount of inflammatory cells in the synovial cavity and synovial tissues, amount of proteoglycan depletion, and the destruction of articular cartilage. Histologic specimens were interpreted by a blinded histopathologist.

Pathologic evaluation of involved joints in *E. coli* asparaginase-treated and control mice revealed a dramatic difference in histopathology. Previously arthritic joints from *E. coli* asparaginase-treated mice demonstrated persistence of some pannus formation, but no destruction of

joint cartilage. In contrast, joints from control mice showed massive destruction of joint cartilage and underlying bone, along with pronounced pannus and inflammatory processes.

Example 15: Enzymatic and Pharmacokinetic Studies

The EC, PEG, and WS asparaginases are purified and biochemical and pharmacological analysis are performed in DBA/1 (H-2q) animals. The enzyme levels in animals treated with these asparaginases are determined in order to correlate efficacy with catalytic activity.

A. Pharmacologic Evaluation of EC, PEG, and WS in DBA/1 Mice.

Pharmacologic analysis of EC, PEG, and WS asparaginases is performed in DBA animals. Plasma L-asparagine and L-glutamine is determined. Administration of asparaginase is correlated with depletion of asparagine and/or glutamine. Neutralizing antisera to EC, WS, and PEG asparaginases is used to establish a cause and effect relationship between immunosuppressive effects of PEG and WS. A WS asparaginase-specific antibody is administered to mice as a negative control for EC asparaginase experiments. The *in vivo* effects of administration of neutralizing antisera to PEG and WS is correlated with plasma amino acid levels and anti-arthritic effects in the DBA mouse model (see above).

Enzyme half-life measurements are performed as follows: Five µL of blood from the tail vein of mice is obtained at specific time intervals after the injection of the particular asparaginase. The 5 µL blood specimen is immediately pipetted into a 0.5 mL of cold 1.19% NaCl in 0.1 M sodium phosphate buffer (pH 7.0) and vigorously vortexed. Blood samples are collected and kept at 4°C until all specimens are collected. For the asparaginase assay, two 0.2 mL aliquots of each time point are equilibrated to 37°C in a water bath. To start the reaction, 0.03 mL of a 0.04 M

L-asparagine solution is pipetted into one of the tubes. The other aliquot receives 0.03 mL of distilled H_2O and serve as a blank. The enzyme reaction is stopped after 60 minutes incubation by pipetting 0.2 mL of 5% TCA into both the reaction mixture and the blank. Tubes are then centrifuged at 5000 *x g*, to remove precipitate. A 0.2 mL aliquot of the supernatant is then be added to 0.2 mL of distilled H_2O, and 0.2 mL of a freshly prepared Nessler's solution is added. Absorbance at 420 nm is determined using a spectrophotometer (Gilford Instrument Laboratories, Oberlin, Ohio).

B. Purification of the WS and EC Asparaginases.

WS and EC asparaginase can be purified to homogeneity as described by Durden, *et al.* in order to characterize these enzymes and compare their biological and enzymologic activities. PEG asparaginase is obtained from Rhone Polec Rorer, Inc. L-asparaginase preparations are shown to be homogeneous by SDS PAGE and free of endotoxin contamination. The efficacy of the PEG asparaginase preparation is also tested in these experiments.

Biochemical analysis of the native WS, EC, and PEG enzymes is also performed, and the Km, Vmax, and substrate specificity of these enzymes are determined. The purity of the enzyme preparations is established by SDS PAGE followed by silver and Coomassie blue staining of gels.

L-asparaginase activity is determined by the amount of ammonia produced upon hydrolysis of L-asparagine (.08 M L-asparagine) using a 0.01 M sodium phosphate buffer (pH 7.0) as the reaction mixture. The assay mixture consists of 10 to 40 IU of a homogeneous enzyme solution diluted to 2.0 mL with 0.01 M sodium phosphate buffer, pH 7.0. Briefly, this assay measures the deamidation of asparagine indirectly by quantitating the release of NH_3 as detected by the Nesslers reagent. A standard curve of NH_4SO_4 is prepared in order to derive an extinction coefficient for NH_3 based on the absorbance at 420 nm. The enzyme reaction is initiated by

the addition of L-asparagine. For Km and Vmax enzyme kinetics, a more sensitive NADPH dependent asparaginase assay system is used.

C. DATA ANALYSIS

Student's t-test is utilized to evaluate the observed differences between asparaginase-treated animals and control DBA animals, the effects of different asparaginase preparations, and different doses of asparaginases.

Example 16: Asparaginase for Treatment of Graft versus Host Disease

A murine bone marrow transplant model for Graft versus Host disease (GVHD)(B6--B6D2F1) (Hill GR, et al. J Clin Invest 102:115, 1998) is used to determine if asparaginase and/or glutaminases can reverse or prevent acute or chronic form of GVHD. This involves the transfer of splenocytes and lymph node cells isolated from C57BL/6J mice to F1 progeny of C57BL/6J x DBA/2J mouse breeding (termed B6D2F1), resulting in bone marrow transplantation across MHC and minor H antigen barriers. In this model, parameters of survival, spleen index, histopathology of liver, skin, small intestine, lung, and spleen are measured with or without asparaginase/glutaminase treatment. This model has shown predictive value in testing agents for treatment of clinically significant GVHD (Kelemen E, et al. Int Arch Allergy Immunol 102:309, 1993).

For these experiments, 13-16 week B6D2F1 mice are irradiated 1300 cGy total body radiation split into two fractions 3 hours apart (^{137} Cs Source). These mice serve as recipients of 60 x 10^6 splenocytes and lymph node cells from C57BL/6J mouse administered by tail vein injection in 0.3 mL of HBSS on day 0 as described (Ellison CA, et al. J Immunol 155:4189, 1995; Ellison CA, et al. J Immunol 161:631, 1998). Mice are monitored daily for toxicity, body weight, and evidence of GVHD. Mice are treated on day +1 with *Wolinella*

or *E coli* asparaginase (50 IU/injection, on Monday, Wednesday, and Friday) for 4 weeks duration.

In another experimental group, mice are treated at time of onset of GVHD with a similar regimen of asparaginase or glutaminase. Splenomegaly associated with GVHD in these mice is monitored in a subset of mice by monitoring total body weight of mice and determining spleen weight. A splenic index (SI) is determined as shown below and spleens are submitted for histopathological analysis.

$$SI = \frac{\dfrac{\text{Spleen wt. (experimental)}}{\text{Total body weight (experimental)}}}{\dfrac{\text{Spleen wt (control)}}{\text{Total body weight (control)}}}$$

Pathological analysis includes examination of H and E stained paraffin-embedded sections of liver, spleen, skin, kidney, lungs, and small intestine for lymphoid infiltration and inflammatory damage to tissues. These are graded according to a histopathological scale as described (Kelemen E, et al. Int Arch Allergy Immunol 102:309, 1993), hereby incorporated by reference herein, including any figures, drawings, or tables. *E. coli* asparaginase can ameliorate the severity of acute GVHD in this model. which one

While embodiments and applications of the present invention have been described in some detail by way of illustration and example for purposes of clarity and understanding, it would be apparent to those individuals whom are skilled within the relevant art that many additional modifications would be possible without departing from the inventive concepts contained herein. The invention, therefore, is not to be restricted in any manner except in the spirit of the appended claims.

All references cited herein are hereby incorporated in their entirety. When used above, the term "including" means "including, without limitation," and terms used in the

singular shall include the plural, and vice versa, unless the context dictates otherwise.